Universidade de Aveiro
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Departamento de Química

Lara Filipa Rocha Andrade

Hemocromatose Hereditária: resposta celular ao *stress* oxidativo

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo Bioquímica Clínica, realizada sob a orientação científica da Doutora Beatriz Porto, Professora Auxiliar do Departamento de Microscopia do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto, e co- orientação científica da Doutora Graça Porto, Professora Catedrática da Universidade do Porto, e do Doutor Pedro Domingues, Professor Auxiliar do Departamento de Química da Universidade de Aveiro

Dedicada aos meus queridos pais, Adriano e Aurora,

às minhas irmãs, Francisca e Beatriz

e ao meu querido namorado Luís

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Doutor Tiago Pereira de Lacerda Costa Duarte

Investigador Auxiliar do Instituto de Biologia Molecular e Celular da Universidade do Porto

Doutora Maria Beatriz Beça Gonçalves Porto e Vasconcelos (orientadora)

Professora Auxiliar do Departamento de Microscopia do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Doutora Maria da Graça Beça Gonçalves Porto (co-orientadora)

Professora Catedrática convidada do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Doutor Pedro Miguel Dimas Neves Domingues (co-orientador)

Professor auxiliar do Departamento de Química da Universidade de Aveiro

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Palavras-chave Hemocromatose hereditária, sobrecarga de ferro, *stress* oxidativo, 1,2:3,4- diepoxibutano, bleomicina, instabilidade cromossómica

Resumo

O ferro é um dos elementos chave para as funções celulares básicas. Se a sua homeostasia não for corretamente mantida, poderá ocorrer uma sobrecarga de ferro no organismo. Os doentes com Hemocromatose Hereditária (HH), com a mutação C282Y no gene *HFE,* possuem uma progressiva e severa sobrecarga de ferro que, se não for tratada, pode levar a dano nos tecidos, podendo mesmo culminar em cirrose hepática e carcinoma. Tendo em conta que o dano tecidular pode estar associado ao *stress* oxidativo (OS) causado pela sobrecarga de ferro, é importante perceber de que modo atua o sistema de defesa contra o OS nas células dos doentes HH com forma severa de sobrecarga de ferro. Poucos estudos foram realizados sobre o potencial estado oxidante nas células do sangue, onde se encontra uma das maiores fontes de reações oxidativas. Contudo, num estudo recente foi demonstrado que linfócitos de doentes com HH, quando comparados com linfócitos de controlos e pacientes com formas secundárias de hemocromatose, apresentam uma maior proteção relativamente à instabilidade cromossómica (CI) induzida por 1,2:3,4 diepoxibutano (DEB) – um agente alquilante que provoca OS. Este estudo sugere uma resposta adaptativa das células HH a níveis elevados de OS. No entanto, ainda não se sabe se esta mesma resposta pode ser observada com outras fontes de toxicidade do ferro, nomeadamente na presença de bleomicina (BLM) cuja atividade depende da formação de complexos com o ferro não ligado à transferrina (NTBI).

Para compreender melhor o estado oxidante das células do sangue dos doentes HH e a suposta resposta adaptativa das células dos doentes de HH à toxicidade do ferro, foi feita a análise de dois parâmetros de OS selecionados: avaliação da depleção da glutationa reduzida (GSH) e da peroxidação lipídica (LPO). Esta análise foi efetuada em eritrócitos (RBC) e linfócitos (LY), tanto no tempo 0 como passadas 36h em cultura, com ou sem indução de OS. O segundo objetivo deste trabalho foi testar se a BLM promove uma resposta adaptativa à CI comparável à que foi observada com o DEB.

Tanto a caracterização dos parâmetros de OS como os estudos de CI foram efetuados em células de 5 doentes com HH, com elevada sobrecarga de ferro, e em células de 6 dadores saudáveis (HD).

Os resultados mostraram que os RBC dos doentes com HH, comparativamente com os dos HD, apresentam uma maior depleção de GSH e maior LPO, quer ao dia 0 quer após 36h em meio de cultura. Estes resultados sugerem um aumento de OS nos RBC dos doentes. Contrariamente, os LY dos doentes de HH apresentaram menor depleção de GSH após 36h de cultura, sendo esta mais notória nas culturas induzidas com DEB e BLM. Adicionalmente, os níveis de LPO são menores em LY dos doentes de HH, após 36h de cultura, comparativamente com os dos HD. Isto sugere que culturas de LY, quer não-tratadas quer tratadas com DEB ou BLM, têm um algum tipo de mecanismo de defesa contra o OS, ainda não compreendido. A frequência de CI induzida por BLM em LY de doentes com HH não é significativamente diferente da observada em LY de HD, não se observando assim uma diferença na capacidade de resposta à BLM, entre células de doentes e controlos. Pode-se então concluir que a toxicidade induzida por BLM não aumenta a CI em células de doentes com HH com forma severa de sobrecarga de ferro.

Keywords Hereditary Hemochromatosis, Iron Overload, Oxidative Stress, 1,2:3,4 Diepoxybutane, Bleomycin, Chromosome Instability.

Abstract

Iron is a key element for basic cellular functions. If iron homeostasis is not maintained it may lead to iron overload. Patients with Hereditary Hemochromatosis (HH) and with the C282Y *HFE* mutation have a progressive severe iron overload that, if it is not treated, may lead to tissue damage, that mostly culminate in hepatic cirrhosis and carcinoma. Having in mind that tissue damage in HH may be related with oxidative stress (OS) caused by iron toxicity, it is important to understand in what way the OS defense is acting in cells from HH patients with severe forms of iron overload. Few studies have been performed concerning the eventual prooxidant state in blood cells, which bear a major source of OS. Nevertheless, in a recent study it was shown that cultured lymphocytes (LY) from HH, when compared with cultured LY from controls and patients with secondary forms of hemochromatosis, have an increased protection against chromosome instability (CI) induced by 1,2:3,4 diepoxybutane (DEB) – an OS-related alkylating agent. This suggests an adaptive response of HH cells to the high level of OS. However, it is not known yet if the same response can be observed with other sources of iron toxicity, namely in the presence of bleomycin (BLM), that acts forming a complex with non-transferrin bound iron (NTBI).

In order to better understand the oxidant status of HH blood cells and the putative adaptive response of HH cells to iron toxicity, a study was performed to characterize two selected OS parameters: evaluation of reduced glutathione (GSH) depletion and of lipid peroxidation (LPO). The study was performed in red blood cells (RBC) and lymphocytes (LY), either basal and after 36h in culture, with and without induction of OS. Induction of OS was performed with DEB and with BLM. A second objective of the present work was to test if the previously observed adaptive response of HH cells to DEB-induced OS can also be observed after induction with BLM.

Characterization of the OS parameters was performed in RBC and LY from 5 HH patients with severe iron overload and 6 healthy donors (HD), at day 0 and after 36h of culture, non-treated and treated with DEB or BLM. Studies of CI were performed in BLM-induced LY from the same 5 HH patients and 6 HD.

The results show that RBC from HH patients, compared with those from HD, have a larger GSH depletion and more LPO, either at day 0 and after 36h in culture medium. This suggests an increased level of OS in HH RBC. On the contrary, LY from HH patients present less GSH depletion after 36h of culture than LY from HD, being this effect more pronounced in DEB and BLM-treated cultures. Additionally, LPO levels were decreased in LY from HH patients after 36h of culture when compared with LY from HD. This result suggests that HH cultured LY, either non-treated or treated with DEB and BLM, have a still not completely understood mechanism of defense against OS. BLM-induced CI in cultured LY from HH patients was not different from the observed in cultured LY from HD. Therefore, we can postulate that toxicity induced by BLM did not increased CI in cells from HH patients with severe iron overload.

Abbreviations

•OH – hydroxyl radical,

BLM – bleomycin

BMP – bone morphogenetic protein

C/EBPα – CCAAT/enhancer-binding protein α

CAT – catalase

CHOP – C/EBP homologous protein

CI – chromosome instability

CREBH – cyclic AMP response element-binding protein H

DCYTB – duodenal cytochrome b

DEB – 1,2:3,4 diepoxybutane

DMT – divalent metal transporter

DNA – deoxyribonucleic acid

DTNB – 5,5-dithio-bis (2-nitrobenzoic acid)

EDTA – ethylenediamine tetra-acetic acid

Fe – iron

GPx – glutathione peroxidase

GR – glutathione reductase

GSH – reduced glutathione

GST – glutathione transferase

H_2O_2 – hydrogen peroxide

HCL – hydrochloric acid

HD – healthy donors

HFE – hemochromatosis gene

HFE – hemochromatosis protein

HH – hereditary hemochromatosis

ICL – inter-strand cross-links

IL – interleukin

IRE– iron-responsive elements

IRP – iron regulatory proteins

KCL – potassium chloride

LIP – labile iron pool

LPO – lipid peroxidation

LY – lymphocytes

MAP – mitogen-activated protein kinase

MHC – major histocomptability complex

NADPH – nicotinamide adenine dinucleotide phosphate

NO• – nitric oxide,

NTBI – non-transferrin bound iron

$O_2•^-$ - superoxide

$ONOO^-$ – peroxynitrite

OS – oxidative stress

PBS – phosphate buffer sodium

PL - plasma

RBC – red blood cells

ROS – reactive oxygen species

RT – room temperature

SEM – standard error mean

SH – thiol group

SLC – soluble carrier

SMAD – Sma and Mad related family

SOD – superoxide dismutase

STAT – transducer and activator of transcription

TBARS – thiobarbituric acid-reactive substance

Tf – transferrin

TfR – transferrin receptor

TLR – toll-like receptor

TS – transferrin saturation

wb – whole blood

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Part I – INTRODUCTION

1 Iron

Iron is a key element for basic cellular functions. It is used as a cofactor for fundamental biochemical activities, such as oxygen transport, energy metabolism and DNA synthesis. Due to flexible coordination chemistry and redox activity, iron allows protein binding, electrons transferring or catalytic reactions mediation (Galaris & Pantopoulos, 2008). However, these chemical reactions induce the production of reactive oxygen species (ROS), and the generation of highly reactive radicals (such as the hydroxyl radical (•OH)) through Fenton chemistry (Koppenol, 1993), which may lead to oxidative stress (OS) and cellular damage (Galaris & Pantopoulos, 2008). Iron levels within cells must be precisely regulated to promote essential functions and provide an appropriate abundance to maintain adequate stores and concomitantly minimize the risk of potential toxicity (Hershko, 2007; Mackenzie *et al.*, 2008). The majority of body iron (at least 2.1 g) is inside the hemoglobin of red blood cells (RBC) used during erythroipoiesis and oxygen transport. Significant amounts are also present in macrophages (up to 600 mg) and in the myoglobin of muscles (~ 300 mg), whereas excess body iron (~ 1 g) is stored in the liver (Olsson & Norrby, 2008) (Figure 1).

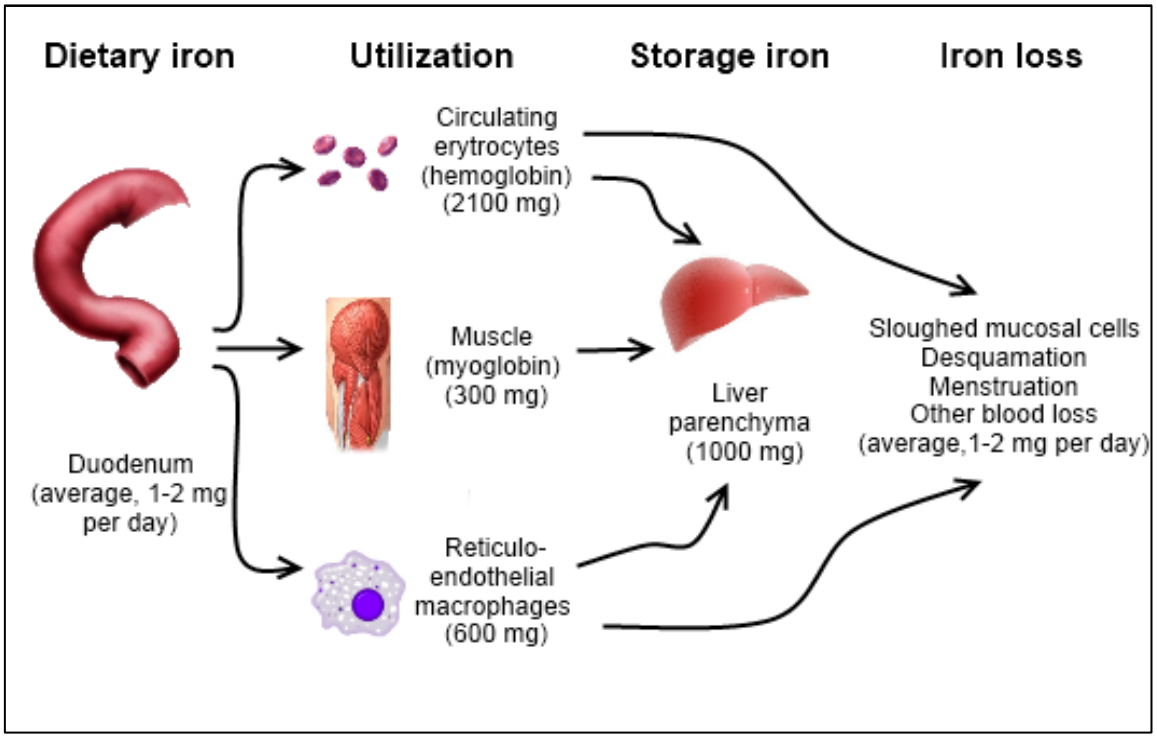

Figure 1 – *Distribution of iron in adults (original figure)*

There are no mechanisms for iron excretion; iron is only lost during bleeding or sloughing of mucosal and skin cells. Thus, balance must be maintained by a rigid control of dietary iron absorption.

1.1 Metabolism of iron

A typical human adult absorbs 1–2 mg of iron per day from the diet (Moyer *et al.*, 2011). Iron is taken up from the lumen of the intestine by the duodenal villus cells involving reduction of Fe^{3+} by ferric reductases, such as DCYTB (duodenal cytochrome b). The subsequent transport of Fe^{2+} across the apical membrane of enterocytes is done by DMT1 (divalent metal transporter 1), a membrane-bound protein transporter (SLC11A2 group). Fe^{2+} is processed by the enterocytes and exported, through ferroportin (SLC11A3 group), across the basolateral membrane into the bloodstream. The ferroportin-mediated efflux of Fe^{2+} is coupled by its re-oxidation to Fe^{3+}, catalysed by the membrane-bound hephaestin or a copper ferroxidase homologous ceruloplasmin present in plasma (Yeh *et al.*, 2009). The Fe^{3+} binds to apo-transferrin to form transferrin (Tf) which circulates in the blood serum and serves to transport iron to other organs, maintaining Fe^{+3} in a redox-inert state (Nelson *et al.*, 2010) (Figure 2).

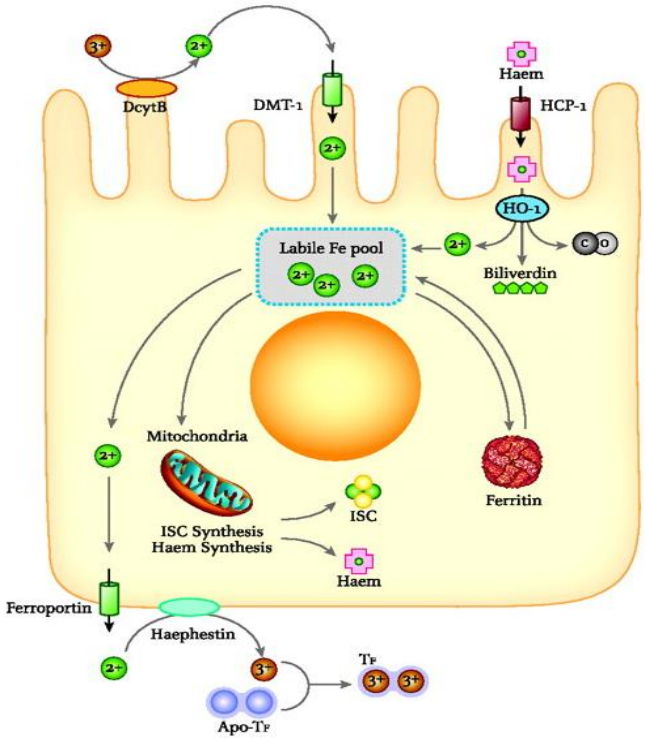

Figure 2 – *Iron import, utilization and export pathways by enterocytes* (Kucukakin *et al.*, 2011)

The ferroportin-mediated efflux of Fe^{2+} is negatively regulated by hepcidin, a liver-derived peptide hormone that binds to ferroportin and promotes its phosphorylation, internalization and lysosomal degradation (Nemeth & Ganz, 2009). Hepcidin accumulates following iron intake and under inflammatory conditions, resulting in decreased dietary-iron absorption and iron retention in macrophages.

Iron activates the expression of BMP6 (bone morphogenetic protein 6) in the liver (Kautz *et al.*, 2008) and intestine (Arndt *et al.*, 2010) which leads to phosphorylation of SMAD1/5/8 (Sma and Mad related family) and translocation of SMAD4 to the nucleus (Figure 3), where it promotes hepcidin transcription upon binding to proximal and distal sites on its promoter (Kautz *et al.*, 2008).

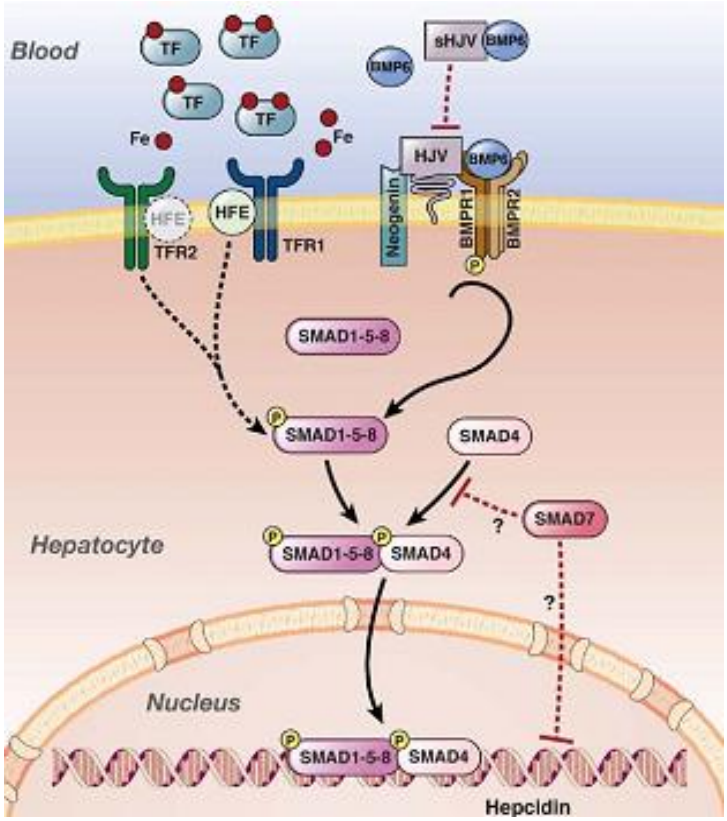

Figure 3 – *The hepcidin iron-sensing machinery* (Pietrangelo, 2010)

Furthermore, basal hepcidin transcription requires C/EBPα (CCAAT/enhancer-binding protein α) (Courselaud *et al.*, 2002). Ramey and co-workers (Ramey *et al.*, 2009) proposed that hepcidin responds to increased Tf saturation by a mechanism requiring a cross-talk between BMP and MAP (mitogen-activated protein kinase) signalling. Supplementary cofactors are needed, including the hemochromatosis protein (HFE), TfR2 and the BMP co-receptor hemojuvelin (Lee & Beutler, 2009). The

cytokine IL-6 (interleukin-6) induces hepcidin transcription via STAT3 (signal transducer and activator of transcription 3) phosphorylation and translocation to the nucleus for binding to a proximal promoter element (Fleming, 2008) while IL-1β activates hepcidin via the C/EBPα and BMP/SMAD pathways (Matak *et al.*, 2009). Stress in endoplasmic reticulum activates hepcidin transcription via CREBH (cyclic AMP response element-binding protein H) (Vecchi *et al.*, 2009) and/or CHOP (C/EBP homologous protein) (Oliveira *et al.*, 2009). In macrophages lipopolysaccharide can also promote autocrine activation of hepcidin through TLR4 (Toll-like receptor 4) signalling (Peyssonnaux *et al.*, 2006). Downregulation of hepcidin involves EPO (erythropoietin) signalling, which promotes the decreased binding C/EBPα to its promoter (Pinto *et al.*, 2008). The cellular storage of iron mainly involves ferritin. One molecule of ferritin provides storage space for up to 4500 Fe^{3+} ions in the form of ferric oxy-hydroxide phosphate. Iron stored within ferritin is considered to be bioavailable and may be mobilized for metabolic purposes(Wang & Pantopoulos, 2011).

The process of cellular iron uptake and storage is regulated by iron regulatory proteins (IRP), cytosolic *trans* regulators able to bind to specific RNA stem-loop structures called iron-responsive elements. The first binding mechanism is an iron–sulphur cluster [4Fe–4S] converting IRP1 to the cytosolic isoform of aconitase. A second mechanism depends on chelatable labile iron pool (LIP) mediated degradation of the IRP1 apoprotein (reviewed in (Wang & Pantopoulos, 2011)). The iron regulates the level of the ubiquitin ligase that is responsible for IRP2 degradation (Nicolas *et al.*, 2003).

LIP is defined as a low-molecular weight pool of weakly chelated iron that rapidly passes through the cell. LIP represents only a minor fraction of the total cellular iron (3–5%). For instance, citrate, phosphate and other organic ions, carbohydrates and carboxylates, nucleotides and nucleosides, polypeptides and phospholipids are some of the ligands with low affinity to iron ions (Marcin, 2003).

If one or more components of the blood iron-sensing machinery fail, iron homeostasis is not maintained, which may lead to an iron overload in some tissues.

1.2 Iron overload and OS

ROS and reactive nitrogen species, such as •OH, H_2O_2, superoxide ($O_2•^-$), nitric oxide (NO•), peroxynitrite ($ONOO^-$), and others, are major sources of OS in cells (Orrenius *et al.*, 2007). When reactive species are present as a result of OS, redox active metal ions localized or bound to the DNA react to form highly reactive •OH in immediate proximity to deoxyribonucleic acid (DNA). •OH then abstracts the 4' hydrogen atom from the deoxyribose sugar backbone, leaving a DNA radical adduct that rearranges, ultimately cleaving the phosphodiester backbone and resulting in strand scission (Perron & Brumaghim, 2009). DNA damage of both types (strand breakage or base damage) can ultimately result in genetic mutations, cancer, or cell death (Jomova & Valko, 2011).

Iron-mediated DNA damage is primarily thought to originate from solvated iron that is not bound to proteins (such as hemoglobin, Tf, or ferritin (Andrews, 2004)**)**. In an iron-overload situation the levels of plasma iron exceed the saturation capacity of Tf, promoting the accumulation of the non-transferrin-bound iron (NTBI). NTBI was shown to promote the formation of free •OH and to accelerate the peroxidation of membrane lipids *in vitro*, consequently leading to OS followed by DNA damage.

Cells contain a large number of protective agents and defense mechanisms to prevent or repair the damage caused by ROS, and also to regulate redox-sensitive signaling pathways. Antioxidants are typically categorized in the following categories: *Small molecule antioxidants* – including both water-soluble compounds, such as Vitamin C or glutathione, and lipid soluble compounds, such as Vitamin E, carotenes, lipoic acid, and Coenzyme Q10; *Large molecule "enzyme" antioxidants* – such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Sies, 1991); *Preventive antioxidants* – including albumin, metallothionine, Tf, ceruloplasmin, myoglobin, and ferritin which delay the formation of new ROS, protecting the essential proteins (Anikó *et al.*, 2007).

SOD is an important endogenous antioxidant enzyme acting as the first line defense system against ROS, catalyzing the dismutation of $O_2•^-$ in to H_2O_2. CAT is usually located in the peroxisome and decomposes H_2O_2 into H_2O and molecular oxygen. GPx is present in the cytoplasm of the cells, and catalyses the reduction of H_2O_2 to H_2O, with the simultaneous oxidation of reduced glutathione (GSH). GSH is a

tripeptide and a powerful antioxidant present within the cytosol of cells and is the major intracellular non-protein thiol (SH) compound. SH groups of GSH react with H_2O_2 and •OH, preventing tissue damage. Glutathione reductase (GR) is a flavoprotein enzyme that regenerates GSH from oxidized glutathione in the presence of NADPH (Nicotinamide adenine dinucleotide phosphate). Vitamins C and E are non-enzymatic endogenous antioxidant present within normal cells that react with free radicals to form less reactive radicals (Siems *et al.*, 2000; Pandey & Rizvi, 2011). Glutathione transferase (GST) is one of the most important antioxidants present in blood cells, which catalyzes the conjugation of GSH with electrophiles (carbon, oxygen, nitrogen). Isoenzymes expression of GST in tissues is very different and sometimes their depletion is associated with various pathologies.

In an iron-overload situation defense mechanisms against OS are essential to prevent the consequences of DNA damage in tissues. Therefore, in disorders presenting very severe forms of iron overload, where tissue damage already occurs, it is important to understand in what way the OS defense is acting.

2 Hemochromatosis

2.1 Disorders of iron overload

Iron storage/overload disorder was first described in 1865 by the French physician Armand Trousseau, from an autopsy of a patient with diabetes presented with "bronze-like appearance". The liver was "granular, of a uniform greyish-yellow colour, and very dense"(Trousseau, 1865). In 1889 the German pathologist Friedrich Daniel Von Recklinghausen proposed the term "hemochromatosis" (Von Recklinghausen, 1889), which was associated to iron metabolism for the first time in 1935 by Joseph H. Sheldon (Sheldon, 1935).

Currently, hemochromatosis is defined as iron overload with a primary or secondary cause. Primary iron overload is associated to hereditary iron overload disorder. The term secondary iron overload is used to describe additional mechanisms which origin an iron overload with no genetic basis: metabolic problems that improve the continuous iron uptake by enterocytes may lead to a high storage into body; a combination of excess iron absorption and red cell transfusions, as well as iatrogenic

iron therapy, can facilitate a secondary iron overload; the end-stage liver disease (Cotler *et al.*, 1998) caused by excess alcohol consumption (Fletcher *et al.*, 2003) and/or chronic hepatitis C (Ludwig *et al.*, 1997) may also cause secondary iron overload . A differential diagnosis of iron overload in Human is shown in Table 1.

Table 1 – *Human diseases associated with iron overload (adapted from (Janssen & Swinkels, 2009))*

Hereditary iron overload	Acquired iron overload	Other
▪ HFE-associated HH (type 1) ▪ C282Y homozygosity ▪ Or C282Y/H63D compound heterozygosity ▪ Non-HFE-associated HH ▪ Type 2A hemojuvelin mutations ▪ Type 2B hepcidin mutations ▪ Type 3 TfR2 mutations ▪ Type 4 ferroportin mutations ▪ Other ▪ H-ferritin IRE ▪ Hemoxygenase deficiency ▪ Neonatal iron overload ▪ Aceruloplasminemia ▪ Congenital atransferrinemia or hypotransferrinemia ▪ DMT1 mutations	▪ Iron loading anemias ▪ Ineffective erythropoiesis ▪ Thalassemic syndromes ▪ Sideroblastic anemia ▪ Myelodysplastic syndrome ▪ Congenital dyserythropoiesis ▪ Increased erythropoiesis ▪ Chronic hemolytic anemia ▪ Parenteral iron overload (including multiple blood transfusions)	▪ Metabolic syndrome ▪ Obesitas ▪ Hypertension ▪ Insulin resistance ▪ Chronic liver disease ▪ Hepatitis ▪ Alcohol abuse ▪ Non-alcoholic steatohepatitis ▪ Porphyria cutanea tarda ▪ Iron overload in sub-Saharan Africa

2.2 Hereditary hemochromatosis

Hemochromatosis, as familiar disorder, was first described by Boulin & Bamberger in 1953 (Boulin, 1953); twenty years later Simon *et al* (Simon *et al.*, 1975; Simon *et al.*, 1976; Simon *et al.*, 1977) confirmed that hemochromatosis is linked to the major histocompatibility complex (MHC) on chromosome 6. The identification of the Hereditary Hemochromatosis (HH) gene came only in 1996, and was located in the short arm of chromosome 6 (Feder *et al.*, 1996), firstly called HLA-H and then renamed HFE (to avoid confusion with an HLA pseudo-gene already called HLA-H) (Bodmer *et al.*, 1997). Nowadays it is known that hemochromatosis gene (*HFE)* encodes an HLA-A class 1-like protein and is located on 6p21.3, (Figure 4) with 4 megabases (Mb) telomeric in the human leukocyte antigen region (HLA).

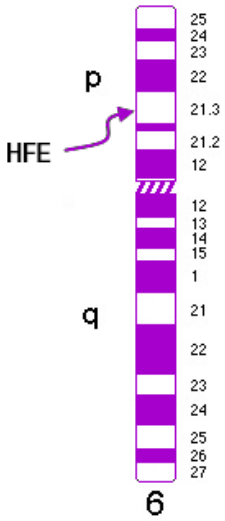

Figure 4 – *Chromosome 6, an approximate location of* HFE *(original figure)*

The *HFE* mutation C282Y is the most commonly found in hemochromatosis, being present in homozygosity in 90 to 95% of all cases, with a prevalence among white subjects of 1:200 to 1:300 (Merryweather-Clarke *et al.*, 1997). It is a single mutation of G to A at nucleotide 845, resulting in the substitution of tyrosine for cysteine at amino acid 282, known as the Cys282Tyr (Feder *et al.*, 1996). This mutation disrupts a disulfide bond that is required for HFE to bind β2-microglobulin and is transported to the cell surface and endosomal membranes, where it interacts with TfR1, forming a complex which is affected by intracellular levels of iron (Schmidt *et al.*, 2008). In the presence of increased saturation of serum Tf, HFE dissociates from TfR1 and is free to bind TfR2. HFE interacts also with TfR2 forming an iron-sensing complex that modulates hepcidin expression in response to blood levels of diferric Tf (Goswami & Andrews, 2006).

Currently, HFE related HH is defined by the presence of C282Y in homozygosity, together with the presence of liver iron overload. In spite of the progressive and severe iron overload, the clinical presentation may be variable, ranging from simple biochemical abnormalities to severe organ damage. Several studies aimed to understand the cause of this variability. In a recent paper it was shown that the setting of $CD8^+$ T-lymphocytes numbers can be a relevant modifier of the phenotypic expression in HH. In fact, in a previous study it was already shown that a large proportion of HH patients have consistently low $CD8^+$ T-lymphocyte numbers correlating with a more severe expression of iron overload (Reimao *et al.*, 1991; Porto

et al., 1994; Porto *et al.*, 1997; de Sousa & Porto, 1998). Low total lymphocyte counts, reflective of low $CD8^+$ T-cell counts, were also shown in HH patients from the north of Portugal (Porto *et al.*, 2001) and from Alabama (United States) (Barton *et al.*, 2005).

The first diagnostic phase of HH is the measurement of serum iron, Tf and ferritin; serum ferritin levels > 200 µg/L in women or > 300 µg/L in men, and transferrin saturation (TS) > 45% in women or > 55% in men, are the criteria for case-finding (Pietrangelo, 2010). TS is the most sensitive parameter for identification of susceptibility for HH (Janssen & Swinkels, 2009). If the TS is increased a genetic evaluation should be done. When *HFE* test is positive, iron overload can be followed by magnetic resonance imaging evaluation – a non-invasive technique useful for identification and management of these disorders. However a negative *HFE* test does not exclude the possibility of a subject developing phenotypic disease. Therefore hyperferritinemia unrelated to iron overload must be excluded as well as secondary iron overload or other disorders such as hepatocyte injury, alcoholic or viral hepatitis, or systemic inflammatory disorders. In these cases, it would be important a liver biopsy histological evaluation.

2.2.1 OS and cancer in HH patients

The most common complications of non-treated HH are hepatic cirrhosis, cardiomyopathy, diabetes mellitus and arthropathy. They may have increased predisposition to malignancy, particularly liver cancer. Up to 30 percent of patients who develop cirrhosis will develop liver cancer, which is the leading cause of death. In addition, some studies have linked HH to nonhepatic malignancies, including esophageal cancer, colorectal cancer, malignant melanoma and lung cancer, although with conflicting evidence. Nevertheless, HH is not associated with an increased predisposition to hematologic malignancies.

It is well established that tissue damage and cancer development in HH is related with OS caused by excessive iron overload. Free iron is a potent promoter of •OH formation that can cause increased lipid peroxidation (LPO) and depletion of chain-breaking antioxidants (e.g. GSH). In fact, iron overload in HH has recently been demonstrated to inflict OS, measured as high levels of the LPO product 8 - isoprostaglandin F2α in urine (Broedbaek *et al.*, 2009). The result can be free radical damage to tissues such as liver (Crawford *et al.*, 2012), pancreas (Cooksey *et al.*, 2004),

heart (Shizukuda *et al.*, 2011), joints (Lynch & Soslau, 2011), and skin. Such oxidative damage in tissues may contribute to the clinical manifestations seen in HH (Franchini, 2006). Antioxidant enzymes such as GSH are a primary defence mechanism for preventing damage by ROS being also important for detoxification of drugs and environmental toxins (Schafer & Buettner, 2001). In iron overload situations, if activity of GSH is reduced, it can result in an increase of OS and hence influence progression of the disease. Therefore, LPO and GSH evaluations may be valuable parameters for the study of OS in cells from HH patients. At present, few studies have been performed concerning the eventual prooxidant state in blood cells, namely in red blood cells (RBC), which bear a major source of iron and in lymphocytes (LY) where the presence of NTBI can be a source of OS.

2.2.2 Chromosome instability in cells from HH patients

Chromosome instability (CI) is defined as a persistently high rate of numerical and structural alterations in chromosomes. It can occur when somatic mutations are not repaired, either by excess of mutagens, mainly ROS, or deficiencies in DNA repair. In HH, iron overload enhances the formation of ROS, and consequently may increase CI, which can culminate in a higher predisposition to cancer development. Most human cancers are characterized by CI. Indeed, when CI involves genes related with chromosome condensation, sister chromatid cohesion, kinetochore structure and function, microtubule formation and dynamics as well as checkpoints that monitor the progress of cell cycle, the regulation of cell proliferation will be affected (Nasmyth, 2002).

Nordenson and co-workers observed for the first time an increase in spontaneous CI in lymphocytes from hemochromatosis patients (Nordenson *et al.*, 1992). At time, the genetic basis of hemochromatosis was not known, so the study population was not *HFE* genotyped. Almost 20 years later, Porto and co-workers observed in lymphocytes from HH patients with C282Y *HFE* mutation also an increase of CI when compared with lymphocytes from HD (Porto *et al.*, 2009).

For the study of pathologic situations associated with increased susceptibility to CI, two approaches can be performed: 1. detection of spontaneous chromosome breaks; 2. detection of chromosome breaks induced by specific genotoxic agents. At present, two agents are used in cytogenetic assays for the study of situations with

hypersensitivity to OS: the antibiotic bleomycin (BLM) and the alkylating agent 1,2:3,4 diepoxybutane (DEB).

BLM is a glycopeptide antibiotic with anticancer properties produced by *Streptomyces verticillus*. It was identified in 1966 (Umezawa *et al.*, 1966), and one of its mechanisms of action is breaking the DNA double helix by the production of free radicals, a process that is oxygen and iron dependent. High concentrations of BLM arrest cells in the G2/M phase of cell cycle, leading to cell death. However, recent studies showed that lower concentrations of BLM induce over-replication (Nakayama *et al.*, 2009). It is commonly used in cytogenetic assays as a radiomimetic agent that causes CI. It is also used for measuring non-transferrin bound iron (NTBI); in the BLM assay, the BLM reacts with NTBI forming BLM-iron complex which releases $OH^{\cdot}$ and promotes DNA damage.

DEB is a genotoxic metabolite of 1,3-butadiene produced by further epoxidation of 1,2-epoxybutane (Vlachodimitropoulos *et al.*, 1997). It is also a bifunctional alkylating agent, being capable of inducing the formation of monoalkylated DNA adducts, DNA cross-links, mainly inter-strand cross-links (ICL) and DNA–protein cross-links. DEB-induced cytotoxicity has been related to oxidative damage. Korkina and co-workers (Korkina *et al.*, 2000) identified, for the first time, the redox-dependent toxicity of DEB. In fact, DEB-induced DNA-DNA and DNA-protein cross-links are a characteristic feature of ROS-mediated damage (Michaelson-Richie *et al.*, 2010). Therefore, DEB is routinely used in CI studies related with prooxidant situations.

In a recent work Porto (Porto *et al.*, 2009) reported that cultured lymphocytes from HH patients have lower CI induced by DEB when compared with both controls and patients with secondary forms of hemochromatosis. This suggests a still not completely understood adaptive response of HH cells to the high level of OS. Adaptive response has already been observed in other situations, in response to a high dose of a cytotoxic agent after repeated low-dose exposure. In order to better explore the adaptive response hypothesis in HH cells, further studies are needed, involving OS-related agents other than DEB. BLM is a good candidate, own to its affinity to NTBI in lymphocytes.

Part II – OBJECTIVES

As previously discussed, tissue damage in HH is described to be related with OS caused by excessive iron overload. Thus, it is important to understand how the OS defense mechanisms are acting in cells of HH patients with severe forms of iron overload. Few studies have been performed concerning the eventual prooxidant state in blood cells. Therefore, the first aim of the present study was to evaluate the OS though measurement of OS parameters in LY and RBC, which bear various sources of OS, from HH patients with severe iron overload. For this purpose: two selected OS parameters (GSH levels and LPO) were determined in RBC and LY from HH patients and healthy donors, at day 0 (basal level) and after 36 hours in culture (non-treated and treated with DEB or BLM).

It has already been described that cultured LY from HH patients have an increased capacity to respond to the genotoxic effect of DEB, which suggests an adaptive response of HH cells to the high level of OS. The second objective of the present study was to test the effect of BLM in LY from HH patients, and compare with the previously observed effect of DEB. For this purpose: CI in BLM-induced LY cultures from HH patients and healthy donors were evaluated. Cultures of 48 and 96 hours were performed, with BLM induction 6 hours before culture harvesting.

Part III – EXPERIMENTAL WORK

1 Subjects

This study includes 5 HH patients (2 males and 3 females with a mean age of 59 years, ranging from 53 to 70 years), all homozygous for the C282Y *HFE* mutation and regularly followed up at the Hemochromatosis Outpatient Clinic of Santo António Hospital, Porto. Patients had, at the time of the study, severe iron overload. A total of 6 healthy donors (HD) (5 female and 1 male with a mean age of 24 years, ranging from 19 to 29 years) were used as controls. In each experiment, control subjects were studied in parallel with patients. The study was submitted and approved by the ethical committee of Santo António Hospital, Porto. An informed consent was obtained from each participant.

For each experiment, 35 mL of blood was collected by venipuncture into vacuum tubes with lithium heparin, from each patient or control.

2 Cells

2.1 Isolation of LY and RBC

LY and RBC were isolated from peripheral blood by a gradient density centrifugation method, using Histopaque solution 1077 in polypropylene centrifuge tubes. Briefly, 3 mL of collected blood was carefully layered on top of 3 mL of Histopaque 1077 in each 15 mL polypropylene tube. The tube was centrifuged at 890 × g for 30 min at room temperature (RT). The LY layer was carefully removed for another tube, using a glass Pasteur pipette, and the remaining RBC layer was removed to another tube. The LY layer was gently mixed with 10 mL of PBS (phosphate buffer sodium) 1X and then centrifuged at 840 × g for 10 min at RT to remove platelets. The supernatant was dispensed and 10 mL of RBC lysis solution (1 M Tris-base HCL (pH 7.2), 5 M NaCl and 1 M $MgCl_2 \cdot 6H_2O$) was added to LY pellet, during 10 min, to lyse any remaining RBC. This suspension was centrifuged at 840 × g for 10 min at RT, after which the supernatant was dispensed. LY pellet was resuspended with 1 mL of PBS.

2.2 Cell preparation for the study of basal OS parameters

In an aliquot with 250 µL of isolated RBC it was added 1 mL of RBC lysis solution during 15 min. This suspension was centrifuged at 180 × g for 5 min at RT.

The RBC pellet was dispensed and the supernatant (hemolysate) was transferred to another aliquot. The hemolysate and isolated LY were immediately stored at -80 ºC for posterior measurement of basal enzymatic antioxidant activities and LPO.

3 Cell cultures

Samples of whole blood (wb) and samples of plasma (PL), where RBC were depleted by gravity sedimentation, were used in the present study. Cultures were set up in RPMI 1640 (Sigma) complete medium supplemented with 15 % fetal calf serum (GIBCO), antibiotics (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin) (GIBCO) and 29 mg/mL of L-glutamine (Sigma). LY were stimulated with 5 µg/mL of phytohemagglutinin (GIBCO) and placed in an incubator at 37°C with 5 % CO_2 atmosphere, for 36, 48 or 96 h.

3.1 Exposure to DEB

DEB ((±)-1,2:3,4-diepoxybutane, [298-18-0], D-7019 Lot 34 H3683, Sigma), prepared in RPMI 1640, was added to appropriate cell cultures 24 h after their initiation, thus exposing cells to the chemical for 12 h. DEB was added at the final concentration of 0,1 µg/mL. Since DEB is a genotoxic agent specific precautions were taken. All culture procedures were handled using appropriate gloves and in a vertical laminar flow hood.

3.2 Exposure to BLM

BLM (Bleomicina TEVA, 15000U/mL) was prepared in RPMI 1640 complete medium. In the appropriate experiments, BLM was added 24 h after the initiation of cell culture at the final concentration of 10 µg/mL. In another set of experiments, BLM was added 6 h before harvesting, at the final concentration of 15 µg/mL.

3.3 Cell preparation for the study of OS parameters after 36 hours of culture

After 36 h of spontaneous and BLM and DEB-induced cultures (either wb and PL), cells were harvested and centrifuged at 180 × g, 5 min, RT. The supernatant of PL-cultures was dispensed. LY pellets were resuspended with 1 mL of PBS. The

supernatant of wb-culture was dispensed. RBC pellets were resuspended with RBC lysis solution (4,5 mL each tube), during 15 min at RT. This suspension was centrifuged at 180 × g for 5 min at RT. The supernatant of RBC was stored in aliquots (1 mL of RBC hemolysate each). Both cell suspensions (LY and RBC) were immediately stored at -80 ºC. OS evaluation was also performed in RBC that were put in culture medium during 36 hours, either with and without induction with BLM and DEB.

4 Characterization of OS parameters

4.1 Sample processing for GSH and LPO quantification

Cell suspension aliquots were de-frozen and spinned at 13 000 × g for 10 s at 4ºC and the supernatants rejected. The pellet was then treated with $HClO_4$ 10 %. After a brief vortexing, the homogenates were centrifuged 13 000 × g for 10 min at 4 ºC. Aliquots of the resulting supernatants (the hemolyse were diluted 20x with $HClO_4$ 5 %) were used for the measurement of LPO and GSH. The pellet of LY was dissolved in NaOH 0,3 M and stored at -20º C for posterior protein quantification.

4.2 GSH quantification

GSH quantification was performed by the DTNB-GR recycling assay, based on the oxidation of GSH by 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB), as described by Vandeputte and coworkers (Vandeputte *et al.*, 1994) with some modifications (Carvalho *et al.*, 2004). $KHCO_3$ 0,76 M (200 µL) was added to the supernatant (200 µL) and centrifuged 13,000 × g, 1 min at 4 ºC. Standard GSH corresponding to concentrations ranging between 0,5 and 15 nmol were also prepared. Freshly prepared reagent solution (DTNB 0,7 mM and NADPH 0,24 mM in sodium phosphate-EDTA buffer, pH 7,5) was added and incubated at 30 ºC during 15 min, in the dark. GR was added immediately before measuring the plate at 412 nm by spectrophotometry. The concentration of total GSH was calculated using a standard curve. GSH levels were measured as µmol of GSH *per* mg of protein present in LY. In RBC, GSH levels were measured as µmol of GSH *per* g of hemoglobin.

4.3 Protein quantification

Protein quantification was performed according to the method of Lowry and co-workers (Lowry *et al.*, 1951), using bovine serum albumin as standard.

4.4 LPO quantification

LPO was evaluated by the thiobarbituric acid-reactive substance (TBARS) methodology (Buege & Aust, 1978). Results are expressed as nmol of malondialdehyde (MDA) equivalents *per* mg of protein using an extinction coefficient (ε) of 1.56×10^5 $M^{-1}cm^{-1}$.

5 Evaluation of CI in LY from BLM induced cultures

After 48 and 96 h days of culture, cells were harvested after a 1 h incubation with 100 μL colcemid® (GIBCO) followed by hypotonic treatment with 75 mM KCl and fixation in 1:3 solution of acetic acid:methanol. Chromosome preparations were made by the standard air drying method.

Cytogenetic analysis was performed on coded slides and an average of 91 Giemsa-stained metaphases (mode = 100, range = 22-100) was observed. To avoid bias in cell selection, consecutive metaphases, which appeared intact with sufficient well-defined chromosome morphology, were selected for the study. Each cell was scored for chromosome number and structural abnormalities. Achromatic areas less than a chromatid in width were scored as gaps; achromatic areas more than a chromatid in width were scored as breaks. Tri-radial and quadri-radial configurations and dicentric and ring chromosomes were scored as rearrangements (Figure 5). Gaps were excluded in the selection of chromosome aberrations and rearrangements were scored as two breaks. As CI parameters, percentage of aberrant cell and nº of breaks *per* cell were used.

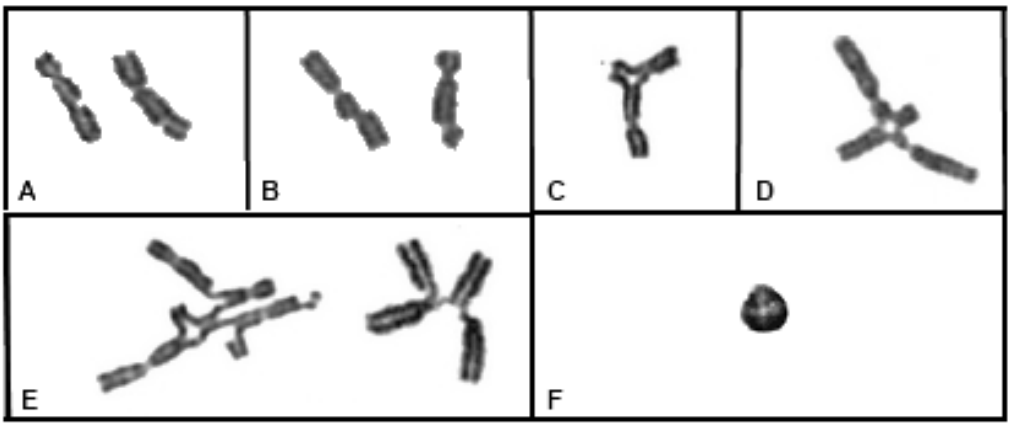

Figure 5 – *Examples of chromosomal aberrations in induced chromosomal breakage:***[A]** *Chromatid gap;* **[B]** *chromatid break;* **[C]** *tri-radial configuration;* **[D]** *quadri-radial configuration;* **[E]** *others figures configurations*; **[F]** *ring chromosome.*

6 Statistical analysis

Graph results were expressed as mean ± SEM. Statistical comparison among groups was estimated using two-way ANOVA, followed by the Bonferroni post hoc test, and comparison between two groups was estimated using unpaired t-test, both with GraphPad Prism, version 5.0 software. *P* values lower than 0,05 were considered as statistically significant.

Part IV – Results

1 Evaluation of the OS parameters

Evaluation of OS in LY and RBC from HH patients was performed, in comparison with LY and RBC from HD, using two selected OS parameters: GSH and LPO levels. These parameters were measured at day 0 (basal) and after 36 h of culture, with and without induction with DEB and BLM. For OS evaluation of RBC after 36 h, these cells were put in culture medium and in wb-culture. For OS evaluation in LY, PL-culture was performed. The results are shown in figures 6-9.

1.1 GSH levels

In both groups (HH patients and HD),a highly significant (P<0,0001) depletion of GSH was observed in RBC after 36 h either in culture medium and in wb-culture, when compared with GSH levels at day 0 (Figure 6A).

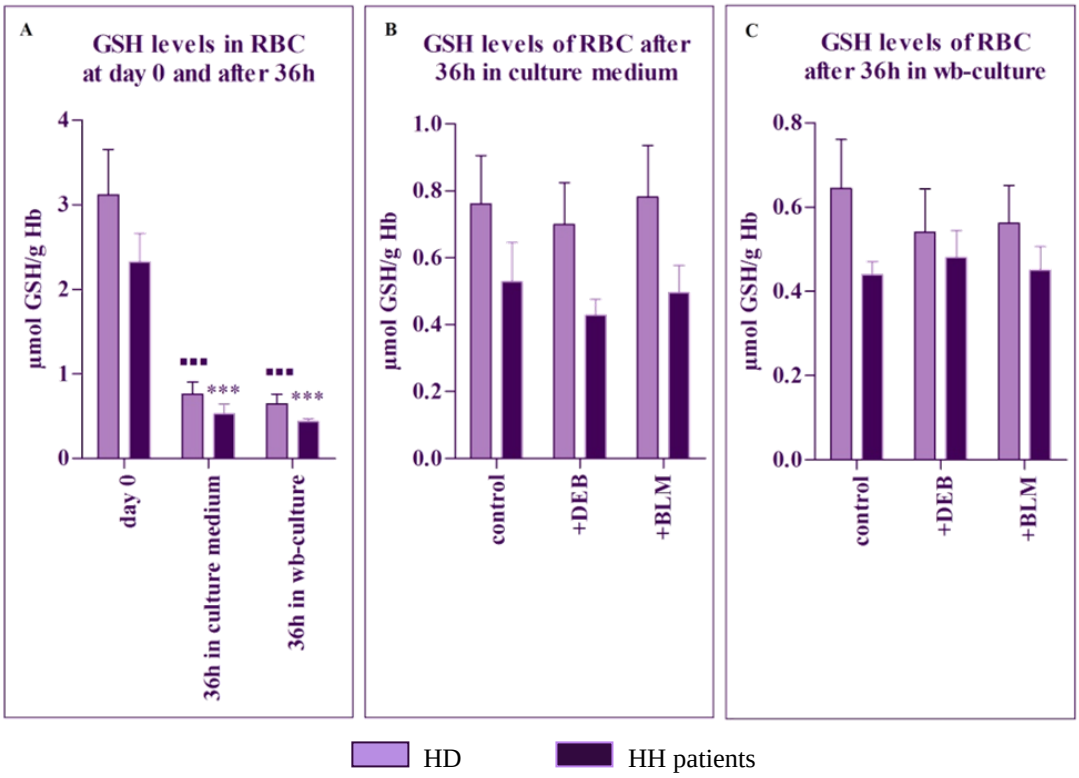

Figure 6 – *Changes in GSH levels of RBC* [**A]** *– at day 0 and after 36 h in culture medium and in wb-culture;* ***P*<*0,001 HH patients RBC at day 0* vs *RBC in culture medium and in wb-culture;* ▪▪▪P*<0,001 HD RBC at day 0* vs *RBC in culture medium and in wb-culture* [**B] –** *after 36 h in culture medium with and without induction with DEB and BLM;* [**C] –** *after 36 h in wb-culture with and without induction with DEB and BLM. Results are expressed as the mean ± SEM in the seven experiments.*

Addition of DEB or BLM had no effect on the level of GSH in RBC from both groups, HH patients and HD. Interestingly, GSH levels were consistently lower in RBC from HH patients when compared with RBC from HD, in all conditions tested: at day 0 (Figure 6A), after 36 h in culture medium, with and without induction with DEB and BLM (Figure 6B), and after 36 h in wb-culture with and without induction with DEB and BLM (Figure 6C). These results are not statistically significant, probably due to the low number of samples tested.

In LY from both HH patients and HD a highly significant (P<0,0001) depletion of GSH was observed at 36 h when compared with GSH levels at day 0, as shown in Figure 7A. Addition of DEB or BLM had no effect on the level of GSH in cultured LY from HD (Figure 7B), but an effect was observed in LY from HH patients, although the results are not statistically significant. Interestingly, GSH levels were consistently higher in LY from HH patients when compared with LY from HD, in all conditions tested: at day 0 (Figure 7A) and after 36 h in PL-culture with and without induction with DEB and BLM. These results are not statistically significant, probably due to the low number of samples tested.

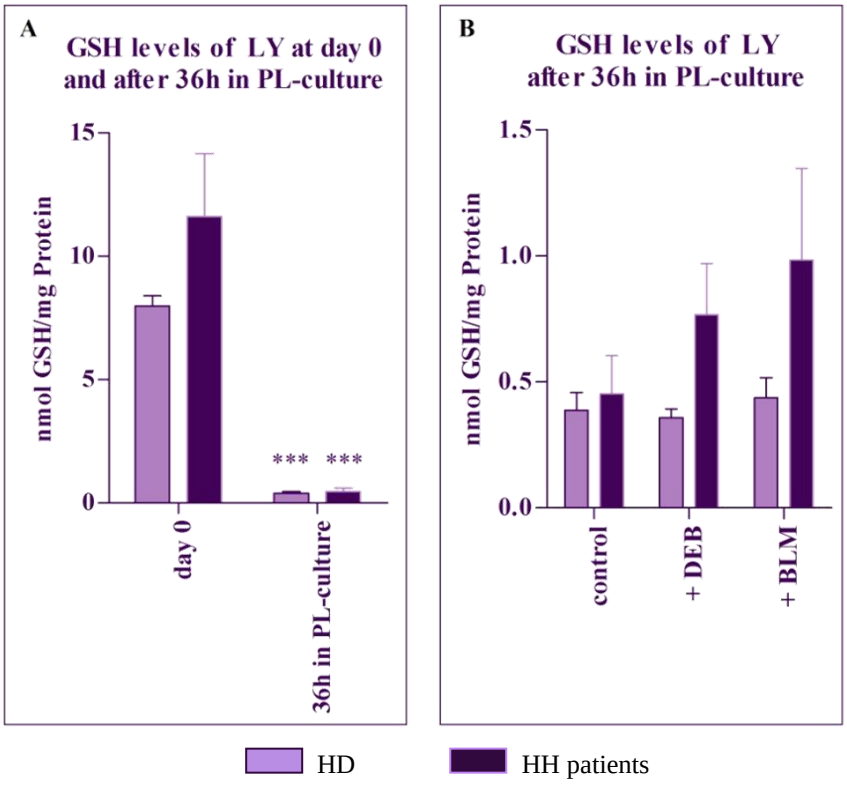

Figure 7 – *Changes in GSH levels of LY* **[A]** *– at day 0 and after 36 h of PL-culture;* ***P*<*0,001* **[B]** *– after 36 h of PL-culture with and without induction with DEB and BLM. Results are expressed as the mean ± SEM in the four experiments.*

1.2 LPO content

As shown in Figure 8A, HH patients exhibited a significant decrease of LPO in RBC after 36 h in culture when compared with the basal level (P<0,05) . This significant decrease was not observed in RBC from HD.

After 36 h in culture medium (Figure 8B), LPO levels in non-treated RBC from HH patients were significantly higher than those from HD (P=0,0225). The addition of DEB or BLM had no effect on the level of LPO, both in RBC from HH patients and HD.

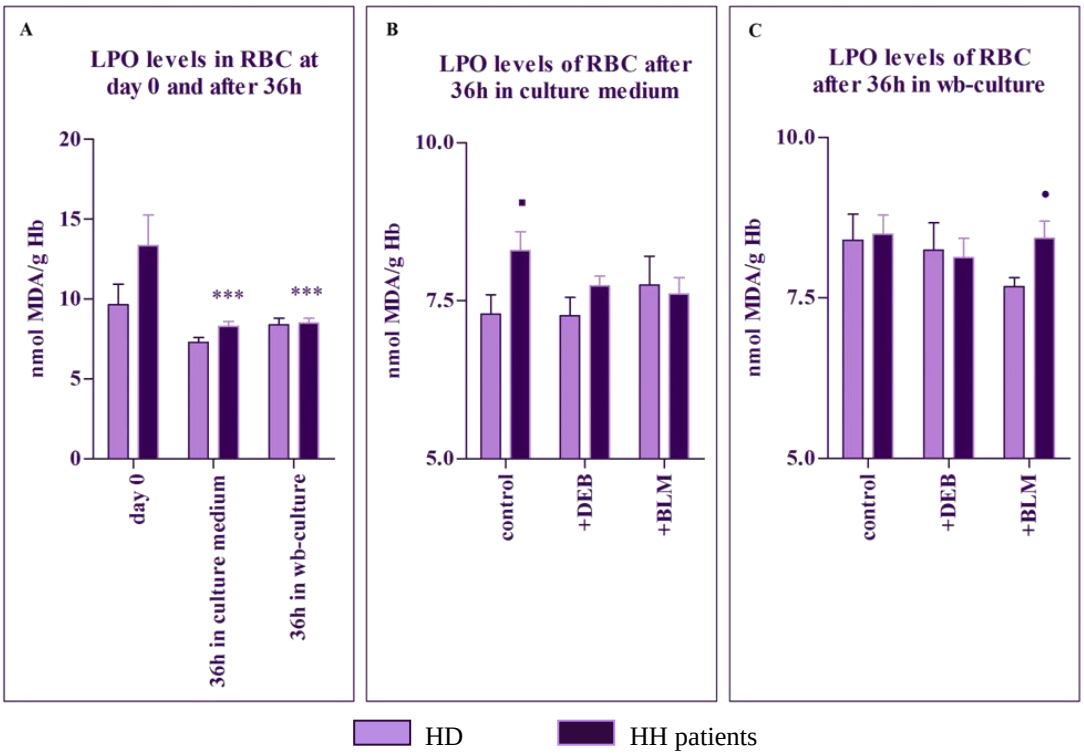

Figure 8 – *Changes in LPO of RBC.* **[A]** *– at day 0 and after 36 h of culture (in culture medium and wb-culture);* *P*<0,05 *HH patients RBC at day 0* vs RBC *in culture medium and in wb-culture* **[B] –** *after 36h in culture medium with and without induction with DEB and BLM;* ▪ *P*=0,0225 *HH patients RBC* vs *HD RBC in control culture* **[C] –** *after 36 h in wb-culture with and without induction with DEB and BLM;* • *P*=0,0130 *HH patients RBC* vs *HD RBC in wb-culture induced with BLM. Results are expressed as the mean ± SEMin the seven experiments.*

After 36 h in wb-culture (Figure 8C), the addition of DEB or BLM had also no significant effect on the levels of LPO, in RBC from both HH patients and HD, except for a significantly higher value observed in BLM-treated RBC from HH patients in comparison to the levels found in HD (P=0,0130).

In LY from both HH patients and HD a decrease in LPO level was observed at 36 h when compared with LPO levels at day 0, as shown in Figure 9A. However, this decrease was only significant in LY from HH patients. After 36 h in PL-culture (Figure 9), the addition of DEB or BLM had no significant effect on the levels of LPO, in LY from both HH patients and HD.

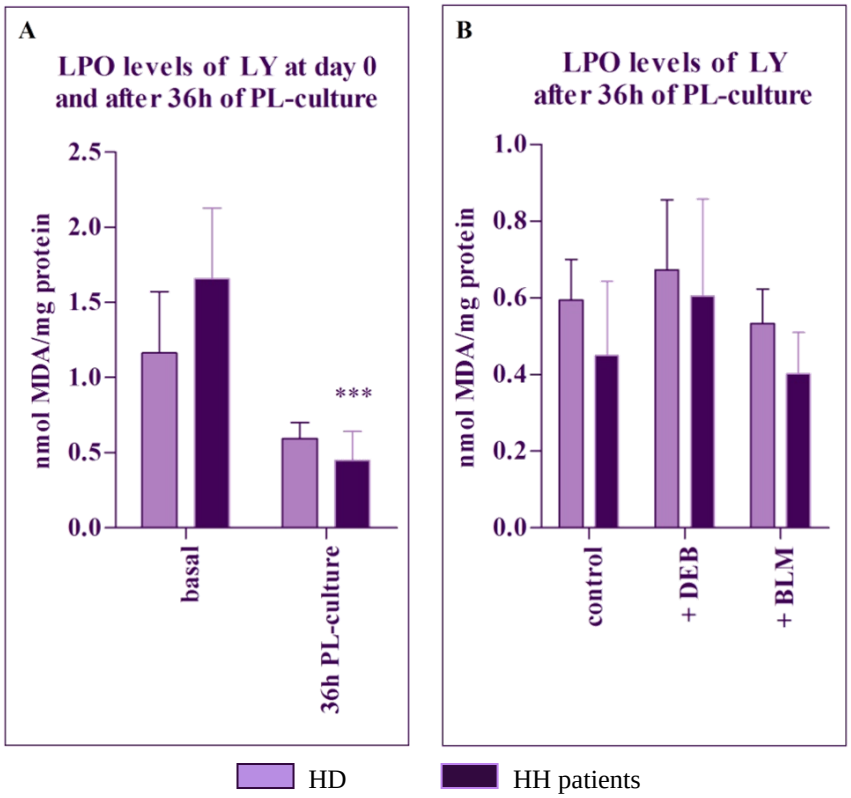

Figure 9 – *Changes in LPO levels of LY* **[A] –** *at day 0 and after 36 h of PL-culture;* $^{*}P<0,05$ *HH patients LY at day 0* vs *after 36 h* **[B] –** *after 36 h of PL-culture with and without induction with DEB and BLM. Results are expressed as the mean ± SEM in the four experiments.*

2 Evaluation of CI in LY from BLM induced cultures

Induced CI in LY cultured in the presence of BLM was measured by two parameters: percentage of aberrant cells and number of breaks *per* cell. The results are shown in Table 2.

Table 2 – *BLM- induced CI in LY from HH patients and HD. 48 h and 96 h cultures were performed with BLM induction during the last 6 h of culture.*

		48h			96h		
	Code No.	***n***	**% Aberrant Cells**	**Nº Breaks *per* cell**	***n***	**% Aberrant Cells**	**Nº Breaks *per* cell**
HD	110	-	-	-	*100*	11	0,26
	117	*100*	15	0,29	*100*	5	0,06
	124	*45*	4,44	0,07	*100*	13	0,16
	133	*100*	6	0,10	*100*	17	0,33
	135	*100*	12	0,25	*100*	7	0,07
	136	*100*	10	0,25	*100*	6	0,14
Mean			*9,49*	*0,19*		*9,83*	*0,17*
Standard derviation			*4,32*	*0,10*		*4,67*	*0,11*
Coefficient of variation			*45,52*	*51,87*		*47,45*	*62,70*
Variance			*18,65*	*0,01*		*21,77*	*0,01*
HH patients	1	*100*	12	0,19	*100*	3	0,03
	2	*22*	13,64	0,14	*50*	10	0,12
	3	-	-	-	*100*	10	0,16
	4	*100*	16	0,26	*100*	15	0,18
	5	*100*	9	0,10	*100*	4	0,06
Mean			*12,66*	*0,17*		*8,40*	*0,11*
Standard derviation			*2,94*	*0,07*		*4,93*	*0,06*
Coefficient of variation			*23,23*	*39,99*		*58,68*	*58,21*
Variance			*8,65*	*0,00*		*24,30*	*0,00*

The first set of experiments was performed with cells from 4 HH patients and 5 HD at 48 h of cultured LY. No significant differences were observed in number of chromosome breaks per cell or in percentage of aberrant cells between HD and HH patients (P>0,05 and P>0,05, respectively). The second set of experiments was performed with cells from 5 HH patients and 6 HD at 96 h of cultured LY. Similarly to the previous cultures, no significant differences were observed in number of chromosome breaks per cell or in percentage of aberrant cells between HD and HH patients (P>0,05 and P>0,05, respectively).

Part V – Discussion

HH is a recessive disorder, characterized by systemic iron overload with consequent tissue damage. The vast majority of HH patients are homozygous for the C282Y mutation in *HFE*, a non-classical MHC class-I gene located in chromosome 6. The mutated protein HFE is unable to bind to the β2-microglobulin present in the plasma membrane of almost cells. This loss of function allows the entry of iron into cell, leading to iron overload.

It is known that iron overload enhances the formation of ROS, with increasing risk of DNA damage as well as increased LPO and protein modifications. The oxidative damage in tissues contributes to the clinical manifestations seen in HH such as fibrosis and later, cirrhosis (Franchini, 2006). Antioxidant enzymes, such as GSH, are a primary defence mechanism for preventing damage by ROS. In iron overload situations, the reduction of GSH activity can result in an increase of OS and hence influence progression of the disease. The relation between GSH depletion and iron homeostasis is not clearly understood so far.

In the present work, selected OS parameters were evaluated in RBC and LY from HH patients and a comparative study was performed with RBC and LY from healthy donors (HD). The two selected OS parameters were GSH and LPO.

1 Evaluation of GSH levels

The increased depletion of GSH in RBC, from both HH and HD, at 36 h, seems to be associated to culture stress. The presence of DEB or BLM did not appear to be associated with increased depletion of GSH. This GSH depletion can occur as a consequence of its function as a substrate in detoxification reactions catalyzed by GPx or GST (Mytilineou *et al.*, 2002). The results of GSH levels showed that RBC from HH patients had more GSH depletion in comparison with RBC from HD. The measurement of GSH in LY showed that there is a significant depletion in the samples from HH comparing these levels at day 0 and after 36 h of LY culture. LY from HH patients seem to respond to the addition of the genotoxic agents DEB and BLM in different ways. Although no significant differences were observed between treatments at 36 h of cell culture, there is an indication of a decreased depletion in GSH when DEB or BLM is added. This may suggest that these patients an adaptive response, but further studies are needed with a larger population.

2 Evaluation of LPO levels

The iron overload present in HH has been also associated to LPO in RBC membrane, composed by polyunsaturated fatty acid, which is extremely sensitive to oxidation (Ferreira *et al.*, 1999). Besides, RBC has more LPO susceptibility, due to continuous exposure to OS (exposed to high oxygen tension, presence of hemoglobin-bond iron). The results of the present work show that RBC and LY LPO levels did not differ significantly among groups. This contrasts with the previous study in HH patients that showed increased levels in HH patients (Niemelä *et al.*, 1999). However, the study was perhaps limited by the relatively small sample size of the groups.

3 Evaluation of CI in LY from BLM induced cultures

Iron *per se* is capable of inducing a wide range of DNA lesions, from base modifications to strand breaks and adducts leading an increase of spontaneous CI (Prá *et al.*, 2012). To test the effect of BLM in the defence against OS, comparatively with DEB (Porto *et al.*, 2009), BLM-induced CI in LY cultures from HH patients and HD was analyzed. The results of the present study showed that both groups had a similar capacity to respond to the genotoxic agent. This effect may reflect the pathways of DNA repair promoted by the different DNA damage of these compounds. BLM is used as chemotherapeutical agent by enabling cell death via ATM/ATR. Whereas DEB, as bifunctional alkylating agent, promotes the majority of the cytotoxity by ICL which subsequently are repaired via Fanconi Anemia Proteins (Andreassen & Ren, 2009).

BLM is a radiomimetic agent that uses Fe^{2+} to attack DNA, which suggests that cells with iron overload may produce a higher level of CI. However, cells from HH patients did not present increased CI. It may be suggested that HH patients may have an increased activity of DNA repair, inactivating BLM, or an increased activity in BLM resistance proteins (Mir *et al.*, 1996), BLM-hydrolase (Bokemeyer, 2008) and DNA polymerase β (Liu *et al.*, 2011). These acting can be related with an adaptive response of HH cells, probably due to continuous high doses of endogenous iron, which activate CI inducers (Tedeschi *et al.*, 1996).

Conversely, the last result may also suggest that HH LY have normal doses of endogenous iron. This hypothesis may explain the low incidence of hemotological malignancies presented by HH patients. To better understand the true meaning of these

results, it would be useful to investigate the role of spontaneous and BLM-induced apoptosis as well as the quantification of proteins involved in BLM inactivation.

4 Comparison of OS parameters and CI in HH

Under GSH depletion, H_2O_2 endogenously produced enhances arachidonic acid release through cellular phospholipase A2 activation in membranes and furthermore, might be converted to •OH by metals such as iron. Excess accumulation of lipid hydroperoxides produced by LPO under intracellular GSH depletion may promote cell death, which may be dependent on the intensity of oxidative stress and lipid peroxidation.

In this study, the apparent protection of GSH depletion in LY from HH patients, after 36 h of culture with BLM and the difference of LPO levels in LY among groups may indicate that LY from HH patients have not increased OS, in comparison with LY from HD. The results of CI induced by BLM in LY from HH patients corroborate these results.

5 Major conclusions of the study

The major findings and conclusions of this thesis are below highlighted:

- No significant differences were observed in OS blood cells response between HH patients and healthy donors. Nevertheless, different responses to OS, measured by GSH levels, were observed between RBC and LY from HH patients.

- BLM-induced LY from HH patients did not show increased CI, comparatively to HD. The decrease in CI obtained with DEB (Porto et al., 2009) was not observed with BLM. The different response of LY to the BLM-induced OS and the DEB-induced OS (Porto *et al.*, 2009) may reflect different pathways of DNA repair, promoted by the different DNA damage of these compounds.

- The results of CI induced by BLM in LY from HH patients corroborate the results obtained through measurement of GSH and LPO, indicating that LY from HH patients have not increased OS, which may suggest normal doses of endogenous iron

6 Future perspectives

Considering all the results obtained in the present work, it is clear that some aspects would benefit from further investigation, in order to clarify several aspects of OS status in blood cells from HH patients as well as their drug metabolization, particularly bleomycin.

Firstly, the size of groups must be increased and secondly, it will be important to evaluate other OS parameters (such as CAT, GPx, GST) to better clarify the true meaning of the results of the present work.

Other challenge to overcome would be the elucidation of DNA repair status in LY from HH patients as well as the quantification of bleomycin hidrolase. This enzyme inactivates bleomycin B2 by hydrolysis of a carboxyamide bond of β-aminoalanine, protecting t cells from BLM toxicity. Recently, Okamura and co-workers suggested that bleomycin hydrolase gene is a suppressor gene in hepatocellular carcinoma (Okamura *et al.*, 2011). HH is associated with an increased risk of hepatocellular carcinoma, so it will be important to understand if LY of HH patients have an increased bleomycin hydrolase activity, which may influence the bleomycin-induced damage in these cells.

This study opened perspectives for future studies, namely regarding the hypothesis that LY from HH patients may have normal doses of endogenous iron.

Part VI - REFERENCES

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Part VII – APPENDICES

Table 1 *Serum iron, tranferrin, transferrin saturation and ferritin levels of HH patients with severe iron overload used in this study***.**

Code no.	Sex	Iron (µg/dL)	Tranferrin (mg/dL)	Transferrin saturation (%)	Ferritin (ng/mL)
01	Male	169	209	57	765
02	Female	159	183	62	371
03	Male	227	182	88	1561
04	Female	165	185	63	1067
05	Female	96	178	38	50

Table 2 *Normal range levels of serum iron, tranferrin, transferrin saturation and ferritin in males and females.*

Sex	Iron (µg/dL)	Tranferrin (mg/dL)	Transferrin saturation (%)	Ferritin (ng/mL)
Male	53-167	200-370	15-45	12,80-454
Female	50-150	200-370	15-45	2,20-178